ОСОБЕННОСТИ ИММУНОЛОГИЧЕСКИ АКТИВНОГО И «ХОЛОДНОГО» ФЕНОТИПОВ ИНВАЗИВНОЙ КАРЦИНОМЫ ШЕЙКИ МАТКИ РАННИХ СТАДИЙ ПО ДАННЫМ СЕКВЕНИРОВАНИЯ ТРАНСКРИПТОМА

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Резюме. Вопросы молекулярной классификации, иммунной гетерогенности и существования различных иммунофенотипов вирус-ассоциированного рака шейки матки (РШМ), в особенности его наиболее ранних клинических и доклинических форм – цервикальных интраэпителиальных неоплазий (ЦИН), – остаются недостаточно исследованными. Цель данной работы заключалась в анализе транскриптомных профилей инвазивного РШМ на начальных этапах его прогрессии, различающихся иммунологическими характеристиками, спектром сигнальных путей и составом микроокружения. Транскриптомный анализ проводился с использованием РНК-секвенирования на платформе Illumina. Панель образцов нативной ткани, полученных в ходе хирургической операции, включала: ВПЧ-положительные ЦИН 1-3-й степени, инвазивный РШМ IA1-IIB стадий и морфологически нормальный эпителий. Транскриптомные профили далее были проанализированы с помощью биоинформатических инструментов, включая поиск дифференциально экспрессированных генов (DESeq2), анализ сигнальных путей (Gene Set Enrichment, GAGE), извлечение клеточного состава (xCell), позиционный анализ дифференциально экспрессированных геномных регионов (PREDA). На первоначальном этапе иерархический кластерный анализ выявил гетерогенность транскриптома образцов РШМ ранних стадий, а именно их распределение по двум кластерам; метод K-means подтвердил наличие трех функционально различных паттернов генов с координированно изменяющейся экспрессией. Сравнительный анализ обогащения сигнальных путей в двух опухолевых кластерах инвазивного РШМ (‘A’ и ‘B’) относительно группы ‘C’, представленной преимущественно ЦИН, показал, что опухолевая прогрессия в кластерах ‘A’ и ‘B’ может основываться на различных иммунных

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механизмах. xCell анализ подтвердил различия в изменении численности популяций иммунных и стромальных клеток, а также суммарных показателей влияния микроокружения, при сравнении кластеров РШМ и ЦИН. По результатам PREDA установлено, что транскриптомные различия ассоциированы с различными хромосомными регионами и ко-локализованы с определенными семействами генов, вовлеченных в регуляцию иммунного ответа. Таким образом, на транскриптомном уровне выявлено существование различных иммунофенотипов РШМ ранних стадий, что может иметь значение для развития методов таргетной и иммунной противоопухолевой терапии.

Ключевые слова: вирус-ассоциированный рак шейки матки, профилирование транскриптома, опухолевая инвазия, опухолевое микроокружение, интраэпителиальные неоплазии, сигнальные пути, иммунный инфильтрат, иммуносупрессия

CHARACTERISTICS OF IMMUNE-ACTIVE AND IMMUNE-SILENT PHENOTYPES OF EARLY-STAGE CERVICAL CARCINOMA AS REVEALED BY TRANSCRIPTOME SEQUENCING

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Abstract. Molecular classification, immune heterogeneity, and the existence of distinct immunophenotypes of virus-associated cervical cancer (CeCa) remain as-yet weakly explored issues, and this is particularly true of its earliest clinical stages and pre-invasive forms: cervical intraepithelial neoplastic (CIN) lesions. The goal of the study was to identify transcriptomic landscapes of invasive CeCa at its initial progression that differ substantially in their immune-related characteristics, patterns of signaling pathways and composition of the microenvironment. Transcriptome profiling was carried out using RNA-sequencing on Illumina platform. A panel of surgical-derived tissue samples comprised human papillomavirus-positive CIN grade 1-3, cancer of FIGO IA1-IIB stages, and morphologically normal epithelium. Transcriptomic profiles were analyzed with the use of bioinformatics tools, such as gene set enrichment (GAGE) for signaling pathways, xCell enrichment for cell composition identification, and PREDA positional analysis of genomic data. Hierarchical clustering revealed heterogeneity of transcriptomic profiles within the early-stage CeCa, namely, the existence of two clusters of tumor samples and three functional patterns of genes showing coordinately altered expression. Pathway enrichment analysis on genes differently expressed between the two clusters/groups of CeCa samples ('A' and 'B') and CIN (group 'C') suggested that invasive tumor progression in groups 'A' and 'B' might rely on immunologically dissimilar mechanisms. xCell analysis confirmed heterogeneity of changes in the abundancies of cell populations when comparing CeCa sample groups and CIN, along with differences in immune and stromal scores. PREDA demonstrated that these transcriptomic differences could be linked to different chromosomal regions and co-localized with particular gene families and potentially the reported virus integration hotspots. Overall, the existence and detectability of different transcriptomic immune-based phenotypes of invasive CeCa at its initial stages of progression is shown, which may provide new options to broaden the knowledge and applicability of target and immune anti-cancer therapy.

Keywords: virus-associated cervical cancer, transcriptome profiling, tumor invasion, tumor microenvironment, pre-invasive lesions, signaling pathways, immune infiltration, immune suppression

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Introduction

According to WHO data on global incidence and mortality rates of oncological diseases and the results of the Global Cancer Observatory database analysis, cervical cancer (CeCa) is the fourth most common cancer in women and therefore continues to be a major health problem [2]. One of the reasons for this may arise from insufficient understanding of pathogenesis mechanisms hampering elaboration of new diagnostic and therapy approaches. Rapid advance in cancer immunotherapy boosts an ever-increasing interest toward CeCa due to its virus-associated nature. Current high-throughput techniques provide researchers with an opportunity to construct immune-based, or tumor microenvironment (TME)-based, classification systems, which serve an essential basis for designing immunotherapeutic approaches [11]. On the whole, classification models proposed by researchers on the basis of expression profiles of immune-related genes and/or immune infiltration patterns are in accord with the concept of "hot", "cold", and "altered" tumors.

It should be noted, however, that the problem of immune heterogeneity and the possibility of detecting diverse immune-related phenotypes have only recently come under discussion with respect to CeCa. By analyzing The Cancer Genome Atlas datasets, some authors define human papillomavirus (HPV) associated CeCa as the "hot" subtype evolving on the basis of chronically inflamed TME and showing high rates of immune infiltration, Th1-dominant cytokine profile, an activated interferon status, high level of genomic instability and hence high neoantigen load [8, 15, 16]. Importantly, high immune activity in a tumor site is believed to invoke intrinsic inhibitory checkpoint mechanisms, which tumor cells take advantage of to combat immune response. However, such categorization of CeCa immunophenotypes presently seems insufficient to account for all the processes of active immunosuppression within this "hot" tumor type, which may be the cause of low-to-moderate efficiency of immunotherapy in CeCa patients [3].

Along with the problem of elaborating CeCa molecular classification and refining its immunophenotypes, there are still many debatable questions regarding the earliest stages of CeCa progression (precancer lesions, microinvasive cancer). Since most CeCa molecular profiling studies addressed more advanced, metastatic and recurrent disease stages, it remains largely unknown how the diversity of CeCa immune "portraits" is formed and what are the putative determinants. Despite the widely studied mechanisms behind the action of HPV-oncogenes, there is still no clear understanding of how an immunologically latent infection transforms into a "hot", heavily infiltrated and inflamed neoplasia in certain cases and why the latter acquires further an immunosuppressive and exhausted phenotype [5].

Addressing the above issues, we performed whole-transcriptome sequencing (RNA-Seq) and bioinformatics analysis of a cervical tissue sample panel consisted of mostly preclinical cancerous and precancerous lesions. We were aimed at showing detectability of distinct transcriptomic patterns that may reflect the formation of different immunophenotypes at early stages of CeCa invasion and progression, as well as gaining deeper understanding of the role of innate immune and proinflammatory pathways in promotion of invasive tumor growth.

Materials and methods

Tissue samples were obtained from patients with high-risk HPV(+) cervical intraepithelial neoplasia (CIN) of grade 1-3 (CIN3 comprised carcinoma *in situ*, n = 5) and early invasive squamous cell carcinoma of the cervix at FIGO stages IA1-II (including microinvasive cancer with stromal invasion < 3 mm in depth, n = 9) during a colposcopy-directed biopsy or surgery; morphologically normal cervical epithelium (n = 2) was also included. The diagnosis was based on comprehensive physical examination, extended colposcopy findings, cytology and histopathology tests, in full compliance with the approved standards for the diagnosis and treatment of patients with gynecological malignancies. All women engaged in this this study were informed and gave voluntary written consent. Cervical tissue samples were placed in a stabilization reagent immediately after excision. Total RNA was isolated using TriZOL (Invitrogen). The quality and quantity of isolated RNA were assessed based on 28S:18S rRNA ratio using Fragment Analyzer system (Advanced Analytical) and NanoDrop-2000.

cDNA libraries were constructed using TruSeq stranded Ribo-Zero kit (Illumina). The adaptors-ligated purified fragments were loaded onto the flow cell using MiSeq v3 sequencing kit; 75 bp end-reads were generated on the MiSeq platform (Illumina). Raw paired-end reads were filtered (sequence quality control was done with the FastQC tool). Then, the

filtered reads were mapped to the reference human genome (GRCh38/p13, NCBI) using STAR aligner to generate BAM-files and, further, calculate read counts. HTSeq package was used to assess the abundance of transcripts in Counts Per Million reads mapped (CPM). Genes with minimum counts of 0.5 in at least one sample were considered for analysis. The generated RNA-Seq datasets has been deposited in the NCBI Gene Expression Omnibus (GEO) with accession ID GSE223804.

The top 1000 most variable genes were selected for hierarchical clustering and heatmap construction. K-means clustering was performed using 2000 most variable genes and 3 clusters considered the most optimal choice. Total transcriptional profiles were compared among the samples via principal component analysis (PCA) along the first two principal components. DESeq2 software was applied to study the differential gene expression. The genes with the base 2 log fold change value |logFC| larger than 1.0 and an adjusted p-value < 0.1 were identified as Differentially Expressed Genes (DEGs). Gene ontology (GO) functional enrichment analysis were carried out on DEGs using Gene Ontology biological processes with an adjusted p-value of < 0.05 and gene count of > 2 considered as the thresholds.

Pathway analysis for patient group comparisons was performed using the Generally Applicable Gene set Enrichment (GAGE) method, and the genes were annotated according to GO biological processes. The minimum and maximum gene set sizes were set to 15 and 2000 respectively, and the pathway significance cut-off was set to 0.2. The top 30 pathways were retrieved for each pairwise group comparison. Identification of co-expression networks and sub-modules was performed using weighted gene co-expression network analysis (WGCNA). Cell-type enrichment analysis of bulk transcriptomes was performed using xCell deconvolution method, and enrichment scores of 64 immune and stroma cell types across samples were obtained. PREDA Position RElated Data Analysis (PREDA) package was conducted to identify genomic regions significantly enriched with upregulated or downregulated genes. The study was performed on the equipment of the Unique Scientific Installation (No. 075-15-2021-665).

Results and discussion

Identification of DEGs and pathway enrichment

To search for the biologically relevant transcriptomic alterations that may reflect the formation of distinct immune-related molecular phenotypes upon transition from a pre-invasive lesion to an invasive tumor, we initially examined distribution of samples between pathologic groups according to their transcriptomic profiles. Unsupervised hierarchical clustering yielded a heatmap with two main clusters that overall matched the expected separation among CIN and cancer groups (Figure 1A, see 2nd page of cover), and PCA confirmed the results. However, it turned out that the tumor group was clearly heterogeneous and could be further subdivided into two sub-groups, which cannot be accounted for by pathological staging.

Therefore, a transcriptomic comparison was next performed between the three groups of specimens identified on the basis of similarity of their gene expression profiles. Groups A and B were designated as “tumorous” (A1-A5 consisted of microinvasive and invasive CeCa only, group B also included one CIN3), while group C (C1-C7) was considered as “control”, since it comprised both Norm cases and most part of pre-invasive CIN cases. K-means clustering approach confirmed successful distribution of groups A, B, and C between the three patterns of coordinately expressed genes; GO enrichment analysis defined the functional differences between these patterns (Figure 1B, see 2nd page of cover).

Pairwise differential expression analysis resulted in 809 down- and 552 upregulated DEG in group A *vs* C comparison, 679 down- and 217 upregulated DEG in group B *vs* C comparison, and 434 down- and 488 upregulated DEG in group A *vs* B comparison, showing a trend of a higher ratio of down-regulated over up-regulated genes in tumor groups. This is, overall, in concordance with the current concept that HPV-dependent carcinogenesis rests on epigenetic reprogramming and silencing of vast regions of the host genome. DEG were found to be enriched in different GO functions for each pair of groups. Group C expectedly exhibited a higher expression level of genes mediating epithelial terminal differentiation. Group A was distinguished by up-regulation of the immune response-related genes involved primarily in the innate immunity reactions and members of cytokine-dependent signaling pathways. Group B showed elevated expression of many genes employed in various forms of (intra-)cellular motility and cardiovascular system-associated processes, whereas group A samples showed up-regulation of genes functionally related to the chromatin structure (DNA packaging and conformational changes accompanying

the cell cycle progression, and response to stress). This provides opportunity to presume that the early-stage A and B tumors may utilize different mechanisms to sustain progression and may thus constitute different phenotypes. In light of this assumption, we analyzed the range of signaling pathways on a more rigorous basis with the use of GAGE approach.

According to GAGE, repression of the epithelial differentiation program was a common feature distinguishing both groups A and B. Besides, it emerged that group 'A' datasets displayed higher prevalence of DNA/chromatin- and immunity-related gene sets (Table 1). Regarding chromatin-associated processes, GAGE reveals these include not only spatial chromatin organization per se (at the level of DNA-histone interactions), but activation of chromatin remodeling and epigenetic regulation (such as post-transcriptional silencing) as well; with detection of DNA damage repair (DDR) signaling no less important. One can suppose the tumor evolution in this case is accompanied with the large-scale epigenetic repression of oncosuppressor genes, with DDR reflecting a higher degree of genomic instability and a mutator phenotype. The observed enrichment in immune-related gene sets most likely implies a primary role of interferon-dependent and other innate immunity mechanisms, which could be elicited by genomic instability and, in turn, could foster the adaptive immunity arm with tumor progression. Similarly, a marked up-regulation of innate immunity pathways (such as DNA sensor-mediated or interferon-induced), along with DDR "heating-up" the immune TME, has been evidenced by other researchers who explored transcriptomic profiles of more advanced CeCa stages using publicly available databases [7, 12, 13].

We found it curious that the early consecutive stages of cervical cancer progression, differing actually in their invasion status, demonstrated activation of a specific set of genes with immune annotations. Activity of a number of these genes is known to be abrogated by HPV-oncoproteins in a chronic infection setting [8, 9]. On the other hand, it is well established that chronic antigen stimulation can turn on inhibitory immune checkpoint mechanisms and lead to immune exhaustion/suppression thereby restraining anti-tumor response. We therefore looked at the expression profile of individual genes recognized as immune checkpoint modulators: indeed, an immune-active group A displayed significantly enhanced expression of several markers (e.g., BTN3A1/2, SLAMF7, TIGIT); an increasing trend was also observable for PD-L2 expression. Unlike A, group B displayed significant elevation of an immunosuppressive HMGB1 gene and CD73, but reduced GZMB expression.

To get closer to the systemic-level understanding of relations between the observed gene expression changes and CeCa phenotypes at initial progression stages, a search for gene co-expression networks (modules) was done with the use of WGCNA. Ten different modules of the highly correlated genes were obtained, and subsequent GO enrichment analysis of these modules pointed out that the mechanisms guiding developmental programs, including angiogenesis, cell adhesion and migration, might be functionally important to the formation of CeCa phenotypic traits at early disease stages; the mechanisms of chromatin conformation maintenance and remodeling turned out to be tightly coupled with the morphogenetic processes. A separate module was enriched for immune effector pathways.

Identification of differentially expressed chromosome regions

The above results suggest that the chromatin structure-associated processes play a role in diversifying the molecular portrait of CeCa. Furthermore, in HPV-dependent cancers, the proximity effect of HPV-integration sites may influence the functional control mechanisms of genome expression [14]. To discern chromosomal patterns of highly or weakly expressed genomic regions specific for groups A and B tumors, we applied PREDA [4] and found that down-regulated DEG tended to group into relatively extended blocks, while up-regulated DEG were more scattered throughout the genome. From a functional standpoint, many of these regions overlapped with tandemly arranged gene families that shared common functions in the innate immune response, inflammation, cell death, invasion, and cell identity. For example, interleukin (2q11-q12), chemokine (4q13), and siglec gene clusters were found to distinguish group A from both B and C.

Several other group A-distinguishing gene families were functionally linked by their role in antiviral response and cytosolic DNA sensing: these are interferon-inducible IFIT family genes (10q23), TRIM gene cluster (11p15), and IFN cluster (19q13). Furthermore, group A-specific genome regions contain collections of genes implicated in inflammasome activity and various forms of inflammatory cell death such as pyroptosis (GSDM cluster, pro-inflammatory

TABLE 1. SIGNALING PATHWAY ANALYSIS BY GAGE APPROACH FOR SAMPLE CLUSTERS 'A', 'B', AND 'C' PAIRWISE COMPARISONS

Direction	Pathways	Genes	adj p-value
'A' *versus* 'C' comparison			
Up	DNA conformation change	243	0.016
	Chromatin assembly	127	0.016
	DNA packaging	158	0.016
	Nucleosome assembly	86	0.023
	Protein-DNA complex assembly	189	0.027
	Response to virus	230	0.051
	DNA repair	419	0.051
	Defense response to symbiont	177	0.062
	Chromosome segregation	248	0.062
	Adaptive immune response	273	0.1
	DNA recombination	225	0.1
	Type I interferon signaling pathway	58	0.14
	Cellular response to type I interferon	58	0.14
	Posttranscriptional gene silencing	116	0.16
Down	Epidermis development	232	0.002
	Epidermal cell differentiation	161	0.002
	Keratinocyte differentiation	125	0.003
'A' *versus* 'B' comparison			
Up	Nucleosome organization	115	9.9e-05
	Chromatin assembly	124	0.00014
	DNA replication-dependent nucleosome organization	19	0.0025
	Protein-DNA complex assembly	184	0.00034
	DNA conformation change	240	5e-04
	Negative regulation of gene expression, epigenetic	78	0.002
	Chromosome segregation	244	0.0015
	Gene silencing	185	0.0031
	RDNA heterochromatin assembly	28	0.011
	Mitotic sister chromatid segregation	132	0.0057
	Chromatin remodeling	155	0.0059
	DNA-dependent DNA replication	161	0.0081
	Nuclear division	329	0.013
	DNA repair	411	0.02
'B' *versus* 'C' comparison			
Down	Keratinocyte differentiation	109	2.5e-05
	Epidermal cell differentiation	144	2.5e-05

TABLE 2. xCELL ENRICHMENT SCORES FOR SUBCLUSTERS ('A', 'B', OR 'C') OF CERVICAL TISSUE SAMPLES

Cell population	A	B	C
CD4+ memory T cells	0.016 (0.001-0.048)*	0.0021 (0.000-0.008)	0.002 (0.000-0.015)
CD4+ naive T cells	0.050 (0.014-0.104)*	0.0343 (0.014-0.089)	0.009 (0.000-0.037)
CD4+T cells	0.021 (0.000-0.044)*	0.005 (0.000-0.019)	0.001 (0.000-0.010)
CD8+T cells	0.024 (0.000-0.043)	0.003 (0.000-0.013)	0.003 (0.000-0.012)
CD8/CD4	1.126	0.667	2.270
Tregs	0.016 (0.000-0.038)	0.018 (0.000-0.054)	0.009 (0.000-0.059)
CD8/Treg	1.481	0.177	0.380
Th1 cells	0.050 (0.000-0.090)	0.004 (0.000-0.018)	0.007 (0.000-0.015)
Th2 cells	0.078 (0.003-0.129)*	0.002 (0.000-0.004)	0.019 (0.000-0.078)
B cells	0.059 (0.000-0.190)*	0.002 (0.000-0.008)	0.000
Class-switched mem B cells	0.025 (0.000-0.084)*	0.001 (0.000-0.003)	0.001 (0.000-0.006)
Macrophages M1	0.018 (0.000-0.044)*	0.003 (0.000-0.011)	0.000
Macrophages M2	0.002 (0-0.006)	0.005 (0-0.015)*	0.001 (0-0.002)
M1/M2	8.530	0.650	0.064
conventional DC	0.002 (0.000-0.001)	0.016 (0.000-0.051)	0.024 (0.000-0.083)
inhibitory DC	0.019 (0.000-0.053)	0.148 (0.120-0.165)*	0.055 (0.000-0.135)
activated DC	0.181 (0.110-0.266)*	0.044 (0.000-0.147)	0.025 (0.000-0.042)
Pericytes	0.049 (0.000-0.134)	0.129 (0.100-0.212)	0.045 (0.000-0.127)
Endothelial cells	0.029 (0.004-0.083)	0.067 (0.025-0.146)	0.029 (0.000-0.072)
Epithelial cells	0.113 (0.030-0.184)	0.052 (0.000-0.100)	0.165 (0.108-0.278)
Keratinocytes	0.071 (0.046-0.093)	0.030 (0.000-0.057)	0.100 (0.066-0.139)
ImmuneScore	0.081	0.018	0.008
StromaScore	0.062	0.158	0.057
MicroenvironmentScore	0.143	0.176	0.066

Note. *, $p < 0.05$ (Wilcoxon–Mann–Whitney test).

caspases-1, caspases-4, caspases-5, and caspases-12, cIAP1/2).

Several chromosome regions specifically expressed in either A or B groups showed functional linkage to invasion, as they contained clustered protease gene families or their inhibitors (e.g., SPINK, KLK, MMP). Group B was distinguished by the engagement of CEACAM gene cluster in 19q13 region, as well as SCCA locus (18q21) containing two apoptotic inhibitors and immune modulators, SERPINB3 and SERPINB4. A number of identified group-specific regions spanned the reported integration hotspots [14] suggesting that the differences in HPV integration breakpoints may guide molecular phenotype determination of early CeCa.

Transcriptome-based analysis of cell population composition

We compared the cell population content between the sample groups defined by similarity of their gene expression profiles using xCell algorithm [1]. Considering infiltrating T cells, substantial differences were derived for the $CD4^+$T subset, with its score showing a significant increase in group A (Table 2). Although the counts of $CD4^+$ naïve T cells were comparably elevated in both A and B groups relative to C, the $CD4^+$ memory T cell score increased only

in group A suggesting that not only $CD4^+$ cells are increasingly recruited at the tumor site, but in contrast to B, group-A tumors exhibited a more efficient immune response.

Regarding Th1/Th2-differentiation, A and B groups showed opposite changes: group A was distinguished by a sharp increase in enrichment scores of both Th1 and Th2 gene sets, this increase being higher for the Th1. In group B, Th1 score didn't alter and Th2 score was dramatically decreased. Tregs showed a clear trend to an increase in both A and B groups pointing at a developing immunosuppression in both cases. A different landscape of alterations was observed for $CD8^+$T cell population: unlike group-A that displayed an abruptly increased signal from the total $CD8^+$ cell population and its na ve $CD8^+$T cell subset, group B had reduced frequencies of those cell types indicating $CD8^+$T cell exclusion.

Interestingly, however, while in group C samples, $CD8^+$T cells were about twofold more prevalent than $CD4^+$T cells, A and B tumors showed a notable decline of the CD8/CD4 ratio. The observed trend also agrees with divergent changes of the CD8/Treg ratio: group A demonstrated an increase of CD8/Treg ratio to the values averagely higher than 1, while this ratio fell to negligibly low levels in group B tumors. Group A also showed a clear enrichment across the B cell-differentiation lineage, and a significant increase in the level of class-switched B cells may indicate that the recruited B cells become actively engaged with a specific antibody-mediated response.

Specific differences between groups A and B compared with C were seen in the abundances of dendritic cells (DC) and macrophages (M), particularly their different functional or polarization states (Table 2). Immature DC (iDC) constituted a substantial proportion of conventional DC (cDC) in the pre-invasive group C, which could be explained from a concept that, by the time of CIN3 establishment, the processes of DC activation become suppressed as the result of prolonged action of HPV-oncogenes. However, in group A the percentage of activated DC (aDC) greatly increased, while that of iDC cells decreased. Conversely, in group B the frequencies of iDC cells were significantly elevated suggesting that these tumors likely exhibit exacerbated impairment of DC maturation, which could be one of the underlying causes of deviating T and B cell responses described above. A relatively high prevalence of aDC-specific gene set seen in group A might be related to the higher rates of DDR-signaling, interferon- and pro-inflammatory patterns, which create a more conducing milieu for DC maturation and favor T/B cell infiltration. As to M1/M2-differentiation, group A and B tumors also represented different phenotypes: group A displayed an apparent M1-polarity, whereas group B showed a significantly increased percentage of M2-macrophages.

Quantities of non-immune cells (epithelial, stromal lineages) indicated that group A tumors corresponded to a more differentiated epithelial phenotype, while group B specimens had a more extensive microvascular network. The combined xCell-scores (ImmunoScore, StromalScore, and MicroenvironmentScore) revealed a detectably higher TME impact in both A and B cancerous groups than in C, but group A demonstrated a significantly increased influence from immune infiltration, while group B conversely exhibited an expanded role of tumor stroma. Given that A and B tumors comprised mainly the earliest invasive stages, these groups can be viewed as not only different immunophenotypes, but likewise as different scenarios of transition from intraepithelial growth toward active invasion with different sources of immune suppression. Overall, these findings highlight the dual, complicated role of immune-activating and inflammatory processes in viral-driven biology of CeCa invasive progression. Overstimulation of protective mechanisms in early periods may be one of the triggers and contributors of CeCa invasion. At the same time, at later, advanced stages this could become manifest in the form of a widely documented immune-active but chronically inflamed and exhausted molecular subtype [6, 10].

Conclusion

In conclusion, we have shown the feasibility of detecting consistent transcriptomic immune-related patterns manifested at different levels (i.e., gene enrichment, signaling mechanisms, co-expression modules, cell type enrichment, genome positioning) for the earliest stages of invasive CeCa progression. These transcriptomic landscapes can be taken as a starting point for further investigation of cervical cancer molecular subtypes and the diversity of underlying mechanisms, this being of potential value in point of developing treatment approaches to preclinical or early forms of invasive cervical cancer, as well as stratifying advanced cancer patients for targeted therapy.

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